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Identification of neuropeptides, *flp-1* and *flp-12* targeting neuromuscular system of rice root knot nematode (RRKN) *Meloidogyne graminicola*

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Abstract:
Root-knot nematodes (RKNs), *Meloidogyne* spp, are found in all temperate and tropical areas, and are among the most damaging plant pathogens worldwide. *M. graminincola* is an economically important root parasite on upland, lowland and deepwater rice. FMRFamide-like peptides (FLPs) play significant role as neurotransmitters or neuromodulators in the nervous system and proposed as one of the important targets for the plant parasitic nematode management. Therefore, for the first time, we have cloned and characterized two neuropeptide genes (*flp*-1 and *flp*-12) from the cDNA of preparasitic second stage juveniles of *M. graminicola*. The *flp*-12 contains putative 22 residue long signal peptide at N-terminal suggesting function as an extra-cellular protein. We have found highly conserved motif LFRGR in *flp*-1. These two *flp* genes could be interesting and potential targets for functional validation to explore their utility for designing management strategies.

Background:
Meloidogyne graminicola is one of the major biotic stresses in rice production. Reported losses to *Meloidogyne* vary with ranges of 12-33%; 28-87% 11-73% and 20-80% (mean 43 ± 7.5%) **[1]**. Approximately 57% of Indian rice is produced in the Indo-Gangetic plain, comprising nearly 12 million ha, which can be sub-divided on physiographic and bio-climatic considerations into four transects in India as well as one in the Pakistan Punjab. The four Indian transects have both individual and shared cropping problems. Each transect suffers a range of biotic stresses of which root-knot nematodes (*Meloidogyne*) are a key issue for three of the Indian transects i.e. a) Indian Punjab, Himachal Pradesh and Haryana (b) Eastern Uttar Pradesh, and Bihar and c) West Bengal. *M. graminicola* is also an important rice pest in several major rice growing areas of South and South-east Asia, causing estimated yield losses of between 20% and 80% **[1-3]**. Due to the inadequacy of the existing management approaches, there is a need to develop economically and environmentally sustainable approaches for which nematode genomics could be highly promising by providing novel targets.

Neuropeptide signaling system has been proposed to be the potential target for the management of plant parasitic nematodes **[4, 5]** due to its critical role in various parasitic activities like host recognition, migration and penetration, secretory activities, alimentation, and reproduction. FMRFamide-like peptides (FLPs) have shown to be widely expressed in the nervous system of root knot nematodes, *Meloidogyne* spp **[6]**. The largest families of neuropeptides in nematodes are the FLPs, which possess a C-terminal Arg-Phe-NH2 signature and play a central role in motor activities. There are 28 *flp* genes reported in *Caenorhabditis elegans* encoding at least 72 distinct peptides **[7].** Likewise, 21 FLPs have been identified in economically important plant parasitic root knot nematode, *M. incognita* **[8].** One or more FLPs are known to co-express indicating that there is an interaction between them. Previous reports in *C. elegans* have indicated that, *flp*-1, 12 and 8 are co expressed and a mutation in *flp*-8 did not have any phenotypic aberration in *C. elegans*. But disruption of *flp*-1 and 12 resulted in various neuro muscular dysfunctions **[9].** Further functions of *flp* genes are regulated by microRNAs (miRNAs) and *flp*-12 was identified as a potential target in pine wood

nematode, *Bursaphelenchus xylophilus* **[10]**. Although studies on role of FLPs on behavior of plant parasitic nematodes are limited, a series of RNA interference studies of *flp* genes in *M. incognita* **[6]** and *Globodera pallida* **[5]** revealed aberrant behavioral phenotypes and migrational abilities. *flp*-12 is predicted to be crucial for the normal muscular function in *G. pallida* and *M. incognita* as it has interfered with the nematode migration in a sand column in response to the root defusates **[11].** A similar effect can be envisaged in *M. graminicola* that will be very useful in designing an efficient management strategy. In view of this, for the first time, we have cloned and characterized *flp*-1 and *flp*-12 from the Indian isolates of *M. graminicola* using orthologous sequences present in *M. incognita.*

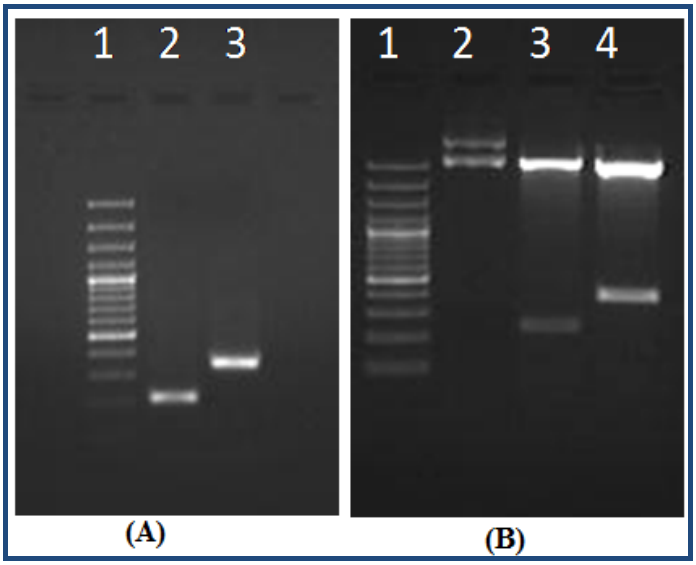

Figure 1: (A) PCR amplification of *flp*-1 and *flp*-12 genes from cDNA of *M. graminicola*. Lane 1:100 bp DNA marker, Lane2: *flp*-1 and Lane 3: *flp*-12; **(B)** *EcoR*I digestion of pGEMT vector containing *flp*-1 and *flp*-12. Lane 1:100 bp DNA marker, Lane 2: undigested plasmid, Lane 3: *flp*-1 and Lane 4: *flp*-12.

Methodology:

Nematode collection

Nematode infected roots were collected from the nematode culture pots and root galls were separated and used for hatching the 2nd stage juveniles (J2s) of *M. graminicola.*

RNA Isolation, quality controls and cDNA Synthesis

Total RNA was extracted from J2s of *M. graminicola* using NucleoSpin total RNA Kit (Macherey-Nagel, Germany). The quality and quantity of each RNA sample was confirmed by using Nanodrop (Thermo Scientific). The RNA samples with 260/280 ratio from 1.9 to 2.1, 260/230 ratio from 2.0 to 2.5 were used for the analysis. One µg of the RNA sample was reverse transcribed to cDNA by using cDNA synthesis Kit (Superscript VILO, Invitrogen).

Cloning and Sequencing

flp-1 *and flp*-12 were amplified from the *M. graminicola* cDNA, using the primers designed based on the published nucleotide sequence of *flp*-1 and *flp*-12 genes of *M. incognita*, (Accession Nos. AAW56944 and AY804187 respectively). The sequences of the primer sets for *flp*-1 were forward primer: 5' ATTCCTGGTCAGATGCAGACCCAA3'; reverse primer: 5'CCACTACTTCGGCCAAATCGAAGA3' while *flp*-12 was amplified using forward primer: 5'CCCAAGTTTGAGCTCTAAAAACAC3' and reverse primer: 5'TCATCGTCCAAATCGAATGA3'. PCR amplification reactions were performed in 50 μl reaction volume containing 5 μl 10 x assay buffer, 200 μM each of dATP, dCTP, dGTP and dTTP (Fermentas), 0.5 μM each primer, 0.5-2 units of Taq polymerase (Sigma-Aldrich) and 1 µl cDNA. The PCR amplification consisted of initial denaturation at 94^{0}C for 4 min, followed by 35 cycles of amplification, denaturation at 94^0 C for 60 s, annealing at 60^{0}C for 30 s and extension at 72^{0}C for 1 min with a final extension at 72^{0}C for 10 min. The amplified product was separated on 1.2% Agarose gel electrophoresis to confirm the size of the amplified product.

Fresh PCR product was cloned into pGEM-T easy cloning vector (Promega, USA) using standard protocol. Freshly prepared competent cells of *Escherichia coli DH5a* were transformed with the recombinant plasmids. Positive clones were selected by blue white colony screening along with ampicillin and colony PCR. Recombinant colonies were used for plasmid extraction. Inserts in the clones were confirmed by restriction digestion with *EcoRI*. The positive clones were custom sequenced by ABI SOLiD sequencing system **[12].**

Bioinformatics Analysis

The amino acid sequences were deduced from the corresponding nucleotide sequences by using the OrfPredictor (ORF-Predictor) server, which is designed for ORF prediction and translation of a batch of EST or cDNA sequences. Database searches were performed with the BLAST Network Service (NCBI, National Center for Biotechnology Information), using the BLASTX and BLASTN algorithm **[13]**. Comparison with the homologous sequences was done with ClustalW **[14]**. Gene ontology term was assigned through AmiGO BLAST **[15]**. Phylogenetic trees were plotted by the maximum likelihood method using MEGA5 software package **[16, 17]**.

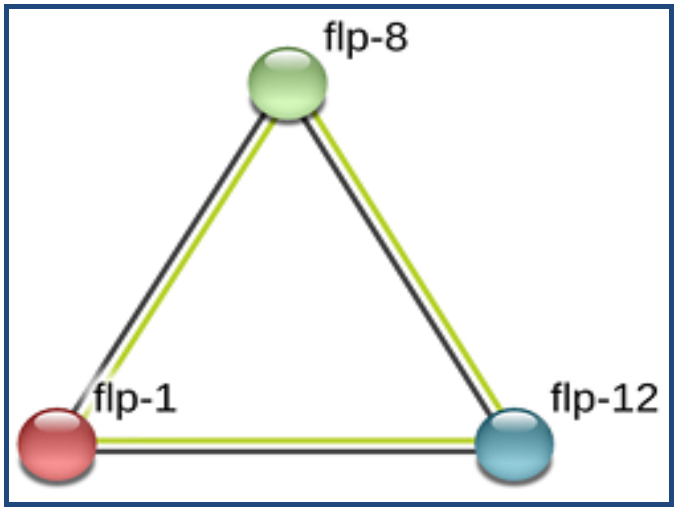

Figure 2: Interaction network generated by the STRING database based on spring model, generated for *flp*-1, *flp*-8 and *flp*-12. Confidence scored for co-expression of *flp*-1 and *flp*-12 (0.730) and confidence score for co-expression of *flp*-1 and *flp*-8(0.822).

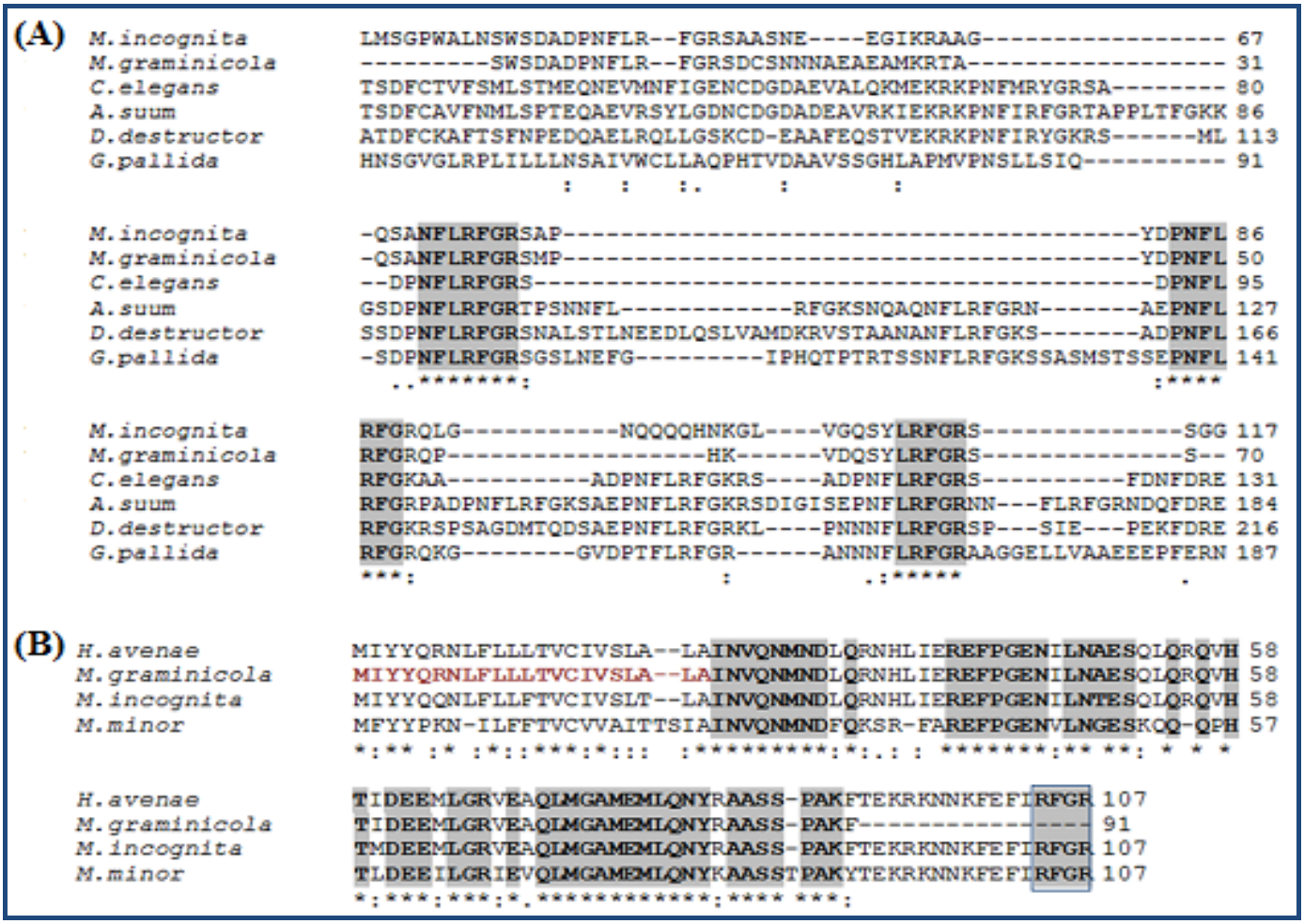

Figure 3: Multiple sequence alignment of the predicted amino acid sequence of the **(A)** *flp*-1 and *flp*-12 **(B)** from *M. graminicola* with *flp* sequences from other nematodes. Genbank identifier for the sequences used of *flp*-1 as follows, *M. incognita* (AAW56944); *G. pallida* (CAC36149); *Ditylenchus destructor* (ACZ97403); *Ascaris suum (*AEX09241); *C. elegans* (NP_872078) and for *flp*-12 genebank identifiers as *H. avenae (*AEN71840*); M. incognita* (AAX19364); *M. minor* (ABB49052) and *G. pallida* (CAC32452) and Signal peptide shown in red.

Result & Discussion:

***Cloning of flp genes from cDNA* of *M. graminicola* and deduced amino acid sequence**

Partial cDNA fragments of 214 bp and 299 bp, named *Mg-flp*-1 and *Mg-flp*-12 respectively, were generated by PCR amplification from *M. graminicola* **(Figure 1A)** cloned into pGEM-T TA cloning vector and confirmed the recombinant colonies. The inserts in the plasmids for the positive recombinants were confirmed by restriction digestion with *EcoRI* **(Figure 1B)**. The cloned genes were sequenced using ABI solid sequencing platform and sequences along with the predicted ORF obtained from ORF-predictor.

Characterization of the sequences

Sequences of the partial cDNAs of *flp*-1 (214 bp) and *flp*-12 (299 bp) obtained from *M. graminicola* were submitted to Genbank sequence database (Accession Nos. KC250005 and KC250006).The percentage of the GC content was 43% and 35% for *flp*-1 and *flp*-12 respectively. BLASTN was carried out to determine the homology against non-redundant Genbank database. *flp*-1 showed best hit with the *M. incognita* having 70% identities (E value 3e-26) and 17% Gaps. Whereas, database searches for the *flp-12* revealed 99% identity with *Heterodera avenae* **[18]** and 95% with *M. incoginta*. Conceptually translated nucleotide sequences into the corresponding amino acids resulted in 70 (*flp*-1) and 91 (*flp*-12) amino acids.

The BLASTX algorithm was also used to compare the *M. graminicola* sequence with protein sequences in database. *flp*-1 showed the most significant match with (Score = 236, Expect = 1e-23) with that of *M. incognita* while *flp*-12 showed most significant (Score = 472, Expect = 6e-59) match with FMRFamide-like protein 12 of cereal cyst nematode, *H.avenae*. SignalP server was used to identify the signal peptide in both the FLPs. SignalP result did not produce significant hits for *flp*-1. However, *flp*-12 was predicted to contain a putative N-terminal short secretion signal peptide of 22 amino acids. This indicates that *flp*-12 could be involved in extra cellular function like signal transduction. AmiGO BLAST hit was performed to assign the putative function of *flp*-1 and *flp*-12 from *M. graminicola* and suggested their potential role in neuropeptide

signaling pathway (GO: 0007218) and locomotory behavior (GO: 0007626).

Putative function for the *flp*-12 sequence was identified as G protein coupled receptor activity (GO: 0004930). Previous studies on *C.elegans* showed that *flp*-1 can encode up to seven distinct, yet highly similar, FMRF amide-related peptides (FaRPs), small neuromodulators peptides that are characterized by a C-terminal Arg-Phe-amide motif. *flp*-1 peptides are required for the regulation of several behaviors, including well-coordinated, sinusoidal movement and the transition between active and inactive states of egg laying. Receptors for the *flp*-1 peptides have not yet been identified, but genetic studies have suggested that *flp*-1 peptides may act through G-protein coupled receptors. *flp*-1 mRNAs are detected at all developmental stages, and a *flp*-1 translational reporter fusion detects expression in the anteriorly positioned neurons AVK, AVA, AVE, RIG, RMG, AIY, AIA, and M5. Based on spring model, generated interaction network image by the STRING database suggested that *flp*-1, *flp*-8 and *flp*-12 are co-expressed **(Figure 2).** *flp*-8 encodes three copies of a FMRFamide-related short peptide neurotransmitter. Although the *flp*-8 peptide can increase pharyngeal action potential frequency, loss of *flp*-8 function does not result in a mutant phenotype, suggesting that *flp*-8 may function redundantly with other FMRFamide-like peptides in the nervous system. *flp*-12 gene encodes a predicted FMRFamide-like peptide neurotransmitter that affects locomotion when injected into *A. suum* **[19]**. Similarly, silencing of *flp*-1 and *flp*-12 of *M. incognita* in our laboratory interfered with host recognition and infection (unpublished data).

Sequence comparison of M. graminicola neuropeptides (flp-1, flp-12) with other nematodes

The conceptually translated Mg-*flp*-1 protein revealed highest similarity with that of Mi-*flp*-1 while the Mg-*flp*-12 exhibited closeness with *H.avenae*. BLASTX identified homologous sequences for *flp*-1 from the free-living nematode *C. elegans*, animal parasitic nematode, *A. suum* and plant parasitic nematodes, *M. incognita*, *D. destructor*, *G. pallida*. On the other hand, homologous sequences for *flp*-12 were only found in plant parasitic nematodes, *H. avenae, M. incognita* and *M. minor*. They were aligned with the deduced amino acid sequence of the *M. graminicola flp*-1 and *flp*-12 respectively, as shown in **(Figure 3).** Multiple sequence alignment result suggested the diversity of *flp*-1 among the homologous sequences. NH2 terminal of *flp*-1 seems to appear poorly conserved than COOH terminal and the LFRGR motif present in three repeats is highly conserved across the nematode species (**Figure 3A**). This strongly suggests that these conserved motifs could be essential in signal transduction mediated by GPCR (G-protein coupled receptor) **[20, 21]**. Alignment of *flp*-12 revealed high sequence conservation only among the plant parasitic nematodes. Figure 3B clearly indicated that C- terminal FMRF signature motif was not amplified during the PCR experiment (As shown in the rectangular box). Phylogenetic analysis was carried out for both Mg-*flp*-1 and Mg-*flp*-12 respectively. Protein sequence for *M. graminicola* and *M. incognita* were clustered together but appeared as an out group. Interestingly, *flp*-1 of *A. suum*, an animal parasitic nematode was grouped with *C. elegans*, a free living nematode. *flp*-1 of *Ditylenchus distructor* and *G. pallida* appeared different from the other sequences **(Figure 4A)**. *flp*-12 of *H. avenae*, *M. graminicola* and *M. incognita* could be clustered together as they were highly similar whereas, *G. pallida* was as an out-group. The sequence from *M. minor* was slightly diverse from the two root knot and one cyst nematode species (**Figure 4B**).

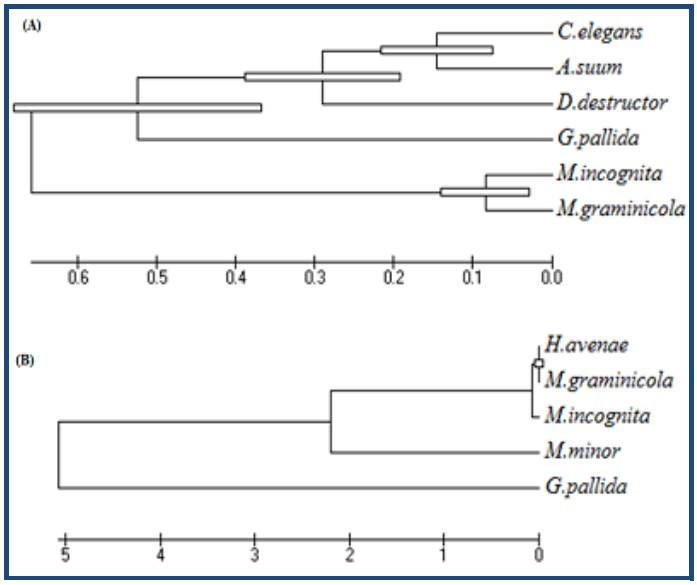

Figure 4: Phylogenetic relationships of the predicted **(A)** *flp*-1 and **(B)** *flp*-12 from *M. graminicola* and *flp* sequences from other nematodes. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. Initial tree for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA5. The homologous sequences genebank accessions were given in Figure 4 were used in this analysis.

Conclusion:

Neuromuscular system of nematodes has been established as an important target for nematode control efforts and majority of the leading anthelminthic drugs act on targets within the neuromuscular system. *M. graminicola* is an obligate parasite of rice; despite of its high economical importance, very less genetic information is available. In this regard, we have cloned and characterized the two partial cDNA sequences of *M. graminicola* neuropeptide, *flp*-1 and *flp*-12 genes. *flp*-1 predicted to have role in neuropeptide signaling pathway and locomotory behavior and putative function of *flp*-12 is indicated its association with G protein coupled receptor activity. These two neuropeptides can be used for future molecular and genetic studies for confirming its practical utility for the management of *M. graminicola.*

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